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RECOVERY OF FILTERABLE FORM OF ANTHRAX BACILLUS FROM ANTHRAX ANTISERA

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RECOVERY OF FILTERABLE FORM OF ANTHRAX BACILLUS FROM ANTHRAX ANTISERA

- USSR -

[Following is the translation of an article by Prof. S. G. Kolesov, and Scientific Associate Yu. F. Borisovich, State Scientific-Control Institute of Veterinary Preparations, in the Russian-language periodical Vet. bull. [Veterinary Bulletin], Vol 24, No 7, 1954, pp 29 - 33.]

SUMMARY

The authors describe seven series of experiments, in six of which they succeeded in recovering filterable forms of anthrax bacillus from anthrax antisera. Their method consisted of cultivating serum on bullion and on agar media, and the microbe colonies obtained were cultivated through several generations on the two media and passed through the organism of white mice, whereupon typical anthrax microbes were obtained, which were tested for virulence on mice, rabbits and guinea pigs. The virulence of the regenerated bacilli did not equal that of the original.

The problems of filterable forms of pathogenic bacteria have been discussed on several occasions recently. However, these reports dealt mainly with microbes evoking contagious diseases of man, such as the agents producing tuberculosis, intestinal typhus, diphtheria, dysentery, etc. There have been certain references to filterable forms of an anthrax microorganism, but these reports have appeared in isolated instances, only.

Scientific data of recent years substantiate the fact that microorganisms subjected to experimental effects and to the effect of the surrounding medium are able to undergo very deep changes in some of their cultural, morphological and virulent properties. In connection with this, the possibility of the formation of avisual forms of microbes may be conceived only if we are on the right track toward the formation of a concept of the factors of deep modification ability and phases of development of microorganisms.

Soviet scientists, starting out from the theory of I. V. Michurin on the possibility of changing the nature of living organisms through directed effects and through the effect of the surrounding medium, attained remarkable achievements in the fields of plant cultivation and horticulture. Some success also has been achieved in respect to directed changes of microorganisms in the fields of industrial, agricultural, medical and veterinary microbiology.

However, these successes still are insufficient, and cannot satisfy the demands of our public economy.

Veterinary specialists in the field of microbiology and epizootology are confronted with urgent tasks in the study of the variability and the phases of development of pathogenic microorganisms under natural conditions and in the organism of animals, which may enable a more complete and correct concept of any given regularities of the course of an infection and of the interrelationship between macro-, and microorganisms. Furthermore, by obtaining filterable forms from microbes subjected to controlled effects, and their regeneration into the original microbial forms it may become possible to obtain weakly pathogenic or apathogenic microbe cultures suitable for vaccine prophylaxis of contagious diseases of agricultural animals.

The development of the indicated problems may be correctly mounted only under the conditions of assumption of deep changes in microbe cells up to the formation of the filterable forms. In other words, the problems of the formation of filterable forms derive from the variability of microorganisms, and from the point of view that obtaining microbe cultures with new properties is admissible in the presence of a factor of formation of filterable forms, with their subsequent regeneration into the microbial forms.

In 1949 G. M. Bosh'yan reported on the possibility of obtaining filterable forms of anthrax microbes from therapeutic anti-anthrax serum.

I. I. Orobinskiy, under the direction of N. I. Leonov (1951), obtained filterable forms from filtrate of Tsenkovskiy vaccine, culture II, and from the precipitating anthrax serum. The authors of the present article also extracted a microbial form of anthrax culture from filtrate of anti-anthrax serum in 1950. Later (1953), V. A. Sergeyev reported on the extraction of anthrax microbes from a precipitating anthrax serum.

This experiment made use of four series of therapeutic anti-anthrax serum, and three series of precipitating anthrax serum.

The methods used in the present authors' detection and regeneration of filterable forms of anthrax microbes were varied. On the whole the consisted of implantation of serum on nutrient bullion and on agar, and after visual forms of microbes were obtained they were regenerated into typical forms through subsequent generations on nutrient media and passage through the organism of laboratory animals. After implantation of the serum on the bullion, a very weak growth appears within various periods of time, in the form of opalescence. Later, the growth becomes stronger and more visible, with the occasional formation of atypical precipitation. Usually, no growth appears on meat-peptone agar. The growth sometimes is in the form of barely perceptible dewey colonies, which are hardly visible in successive transplantations.

In the investigation of MPB first growth cultures a fine granulation in the form of individual particles and conglomerates is observable under the microscope, which do not stain by the Gram method. After many transplantations of the MPB, forms similar to cocci, thin rods and short chains, consisting of bacilli more or less typical of anthrax, are observed in addition to the granular formations. Restoration of the morphologically typical serum culture is best obtained after passage through the organism of white mice. However, it is possible to obtain visual forms of anthrax immunosera with only one transplantation.

First Experiment. In performing experiments on the controlled variability of anthrax microbes in the course of pursuing another research theme, we utilized non-preserved

anthrax serum of the SL series, prepared by the Tabakhmel'skiy Biokombinat in August 1949. The serum for these purposes was filtered through sterilizing filter plates. Sterility of the filtered sera was tested by implantation on bullion and on agar. The implantations were kept in a thermostat for a perion of 10 days.

In one of the experiments, implantations of this type accidentally were left in the thermostat for 20 days, and typical growth of anthrax cultures was observed in them. Microscopic examination revealed that preparations of this culture contained typical anthrax bacilli and filaments. A similar growth in transplantations of serum filtrates was found in two instances. Reproduction of this culture through transplantation to bullion and to agar could not be obtained because the culture was unable to ensure growth with the application of ordinary methods. Innoculation of white mice with a dosage of 0.3ml resulted in survival of five of the animals. An experiment involving control innoculation of these mice with Tsenkovskiy Vaccine I showed that after 10 days the regenerated anthrax culture had no immunological properties.

As mentioned above, the observations described were made in February 1950. We considered it possible to repeat the experiments on regeneration of the filterable forms of anthrax immunosera at a later date.

Second Experiment. In December 1951 it was undertaken to obtain visual forms of anthrax microbes from non-preserved anti-anthrax serum of Series SB, prepared by the Azbiokombinat on 3 October 1949, by implantation of 0.3 to 0.5 ml quantities in test tubes containing meat-peptone bullion. On the 22nd day a culture growth was obtained in the form of a precipitate, easily distinguished in the homogeneous suspension. Microscopic examination revealed fine granular forms and conglomerates of these forms in the preparations obtained from the cultures. Simultaneously with these formations we observed rarely-encountered paired granules and short, thin bacilli, singly and in pairs.

Passage of the indicated cultures through white mice enabled typical forms of anthrax microbes to be obtained from them.

It must be mentioned that the typical microbial forms were not obtained immediately, but through a gradual trans-

ition of the granular forms into coccic, or fine bacillary forms. Very atypical coli-form microbe cultures were obtained from the majority of the mice. However, together with the latter, anthrax cultures also were obtained from individual mice.

Testing showed that the first generations of the SB culture obtained evoked marked edema in white mice. The subsequent generations of this strain, however, lost this property, although they increased in virulence. Thus although a culture of the first passage through the mouse organism was virulent only with respect to mice, and guinea pigs survived innoculation, all the guinea pigs died after innoculation with cultures passed through the organism of white mice four times. Three rabbits innoculated with a dosage of 2 ml of 2-billion concentration of the above culture survived. Upon innoculation of rabbits with anthrax virus it was found that 16 days after innoculation the SB strain has marked immunogenic properties, protecting all three rabbits against infection. In a repeated test experiment of immunogenic properties the strain protected two of four innoculated rabitts, while all control rabbits died.

In a cultural relationship the SB strain has the property of typical growth on MPA and in MPB. However, the morphology of the microbes still was not sufficiently stabilized because granular forms of bacillie, strongly curved filaments or thickened bacilli with a bulge in the center are encountered.

Third Experiment. Visual forms of anthrax microbes were extracted from anti-anthrax serum of Series No 6. The serum was produced by the Azbiokombinat on 11 October 1947, with 0.5 percent phenol additive and heating to a temperature of 58 degrees Centigrade for two hours for purposes of preservation. As a result of the serum implantation in six test tubes with bevelled agar and six test tubes containing bullion, two small colonies appeared on the seventh day in one of the agar test tubes. One of the colonies had the R-form typical of anthrax, and the other had an atypical S-form. Following transplantation of these colonies typical anthrax cultures were obtained in both cases; a multitude of R-form colonies on agar, and growth with clarification of the medium and formation of precipitate in the bullion.

Implantations of extracts of the spleen of dead mice which had been innoculated with this serum also produced an

atypical anthrax culture. In addition to the anthrax colonies, coli-form colonies were observed, containing fine Gram-negative, slowly moving microorganisms.

With careful study of regeneration of anthrax culture in 10-fold passage through nutrient medium and four-fold passage through the white mouse organism it was established that the virulence of the culture did not increase, and it remained avirulent to guinea pigs and rabbits. Most of the white mice innoculated with 0.2 ml of one-day agar culture died within two to four days, with anthrax culture and coli-form culture extracted from the blood of the heart of the mice which died.

In morphological and cultural respects the strains extracted from the anti-anthrax serum of Series No 6 corresponded to the typical anthrax culture.

In the fourth experiment with therapeutic anti-anthrax serum of Series No 64, produced 13January 1948 by the Chita Biofabrika (the serum was preserved with chinosol and was heated to 60°C for two hours) a negative result was obtained, because we were unable to extract an anthrax culture, despite application of the method used in the previous experiment.

Fifth Experiment. Anthrax serum of Series No 6, produced 27 March 1950 by the Tobol'sk Biofabrika and preserved with 0.5 percent phenol and filtered through sterilizing filter plantes, produced no growth after precipitation and implantation on NPA, although growth appeared in NPB after three days. Microscopic examination revealed granular forms of microbes and conglomerates of these forms. Four white mice were innoculated with a culture of the second transplantation in NPB, containing a small amount of atypical fine bacilli in addition to the granular forms. All the mice died within two, three or four days, and typical anthrax culture and coli-form cultures were obtained from their hearts and spleens. Two guinea pigs were innoculated with 1 ml of 2-billion concentration of the anthrax culture extracted from the dead mice, and one of the guinea pigs died of anthrax after seven days, with the second surviving. Three rabbits were innoculated with 1 ml, 2-billion concentration doses of a one-day agar culture extracted from the dead guinea pig. The local reaction to injection of the culture was very slight. The rabbits survived for 18 days, when they were innoculated with anthrax virus. The control rabbits, which had not been innoculated, died of anthrax on

the second, third and fourth days, but only one of the innoculated rabbits died.

In later experiments, anthrax culture was subjected to four-fold passage through the organism of white mice for the purpose of increasing the virulence of the strain. It was established that death of the mice occurred at an earlier date upon each successive passage. As a result of the passages it was noted that the strain increased its virulence not only to white mice, but also with respect to guinea pigs, because all the guinea pigs innoculated with the indicated strain (two experiments were conducted) died of anthrax. However, we did not succeed in strengthening the strain to the original virulence because the rabbits innoculated with it survived. In testing the immunogenic properties of the strain subjected to passage it was found that the strain in 1 ml of four-day agar culture, 2-billion concentration dosage, protected two of the four rabbits innoculated, although all the control animals died. In a third control test of immunity the strain protected one rabbit of four innoculated with the earlier dosage, although all the control rabbits died.

In a culture-morphological respect the strain has the properties typical of anthrax microbes. The growth in MPB is characterized by clarification of the bullion and the formation of flocculation at the bottom of the test tube, and by R-form colonies on MPA. Microscopic examination usually reveals typical anthrax bacilli, and long filaments consisting of bacilli. The strain has good spore-forming properties.

Sixth Experiment. After implantation of precipitating anthrax serum of Series No 34, produced by the Tobol'sk Biofabrika on 17 September 1951 (preserved with 0.5 percent phenol and filtered through sterilizing filter plates), a weak growth appeared in MPB after seven days, and there was no growth in the agar. Under the microscope the presence of granular forms and conglomerates of these forms, plus a small amount of cocci-form formations were observed. Of five white mice innoculated with the culture obtained, 1 died after 24 hours, and two died after seven days. An anthrax culture equal to the coli-form colonies was extracted from the dead mice on an MPA base. The anthrax culture extracted again was injected into four white mice. All the mice died within two or three days, with the formation of very pronounced anthrax edema at the site of injection of the culture. An anthrax culture and coli-form microbes were obtained from

the organs of the dead mice. The anthrax culture obtained from the second passage through white mice was injected into two guinea pigs, one of which died after nine days. Subsequent innoculation of white mice and guinea pigs indicated that the virulence of the culture was not increased as a result of passage through the organism of the white mice. In all the experiments the culture resulted in the death of at least some of the white mice, and did not produce death of guinea pigs. No edema formation was observed in the subsequent experiments upon innoculation of white mice with the strain.

Rabbits innoculated with 1 ml, 2-billion concentration of one-day culture survived. In two control tests of immunity it was found that the strain of anthrax culture extracted from precipitating serum of Series No 34 had weak immunogenic properties, protecting two of six rabbits innoculated with a virulent anthrax culture, while all the control rabbits died.

In testing the culture-morphological properties it was established that the extracted strain has properties typical of anthrax microbes.

Seventh Experiment. In investigating freshly prepared precipitating anthrax serum of Series No 2 a negative result was obtained because we were unable to regenerate the anthrax culture from the indicated serum. It must be noted that the given serum was obtained by another method: through hyperimmunization of producer-horses with live, low-virulence anthrax culture. The preceding series of precipitating sera was obtained through hyperimmunization of the producer with virulent strains of anthrax culture processed with 0.3 percent formalin.

It is difficult to define the influential factor in this case. It may be that we did not succeed in regenerating the microbes because the transplantations and passages through the white mouse organism were not performed correctly, and it may be also, that this depended upon the age of the serum, because we noted that the older the serum the easier it was to regenerate it from the avisual, to the visual form.

We recently set up repeated experiments on the extraction and regeneration of filterable forms of anthrax microbes from three series of therapeutic, and three series of precipitating anthrax sera. As a result, filterable

forms were obtained and were regenerated into visual forms of anthrax microbes from Series 2 therapeutic serum prepared by the Chita Biofabrika on 3 June 1952, and Series 93 precipitating serum produced by the Tobol'sk Biofabrika on 19 August 1949. The virulence of the culture was not strengthened by four passages through the organism of white mice, because guinea pigs innoculated with it survived.

Conclusions

It is evident from the foregoing data that anthrax immunosera (therapeutic and precipitating) contain viable avisual forms of anthrax microbes. This is supported by the fact that we extracted strains of anthrax culture from four series of therapeutic anti-anthrax serum and three series of precipitating anthrax serum at various times after production.

Therefore, the data of our experiments indicate that therapeutic and precipitating anthrax sera contain filterable forms of microbes, by virtue of which they are obtained in the process of hyperimmunization of animals.

In the process of regeneration of avisual forms into visual forms it was established that the ontogenesis of the anthrax microbes is not monomorphic, but pleomorphic.

The content of filterable forms of anthrax microbes in the therapeutic and precipitating anthrax sera is supported by the fact that two series of precipitating, and one series of therapeutic serum were subjected to sterilizing filtration in the course of production.

The question arises of the role of the filterable forms of microbes in the therapeutic and precipitating anthrax sera in the course of their practical application. The problem of the harmlessness of therapeutic serum in its application to animals is especially important in this connection. In the opinion of the present authors the filterable forms of microbes cannot constitute a danger in therapeutic anti-anthrax serum because they already are in the second stage of regeneration, i.e. when they acquire typical microbial form they do not have a virulence which may constitute a danger to the animal organism. Of five strains of anthrax culture extracted by us from therapeutic and precipitating anthrax sera only 2 strains had become strength-

ened to the stage of being virulent to guinea pigs (but not to rabbits), and only after repeated transplantation in nutrient media and passage through the organism of white mice.

Of theoretical interest is the problem of the stability of visual and avisual forms of anthrax microbes under the effect of so-called bactericidal agents and the hyperimmunized organism of the producer.

Thus an antigen applied for hyperimmunization of horses in the course of obtaining precipitating anthrax serum is a bacillary anthrax culture (10-, to 12-hour cultivation), which is "killed" with 0.3 percent formalin, and after testing for virulence on guinea pigs, is injected in the producer-horses. However, as is apparent from our experiments, an anthrax microbe culture may be extracted from precipitating anthrax serum, which after passage through the organism of the white mouse becomes virulent to guinea pigs. Thus neither the effect of formalin nor the effect of the hyperimmunized organism of the producer eliminates the viability of the avisual and visual forms of anthrax microbes. We may not come to the conclusion from this, however, that the microorganism is "deathless," because this is challenged by the advocates of variability. From these new proposals concerning the variability and stability of the microbial forms we may make the sole conclusion that the factors of very deep variability of microorganisms, up to the formation of filterable forms, and the limits of stability, i.e. the viability of avisual, and thus including visual forms of microbes, extend much farther than previously had been established in science.

At the present time investigators still lack data on the size and shape of filterable species of microbes. This problem apparently will be resolved with the aid of electron microscopes.

The existing position on the sterility of anthrax immunosera (in view of a lack of filterable forms of anthrax microbes in the serum) obtained from producers as a result of extended and massive hyperimmunization must be reviewed from the theoretical point of view. The theoretical view that anthrax microbes introduced into the organism of a hyperimmunized producer are destroyed by the action of antibodies and by the cells of the organism also must be reviewed.

The data of our experiments indicate that anthrax microbes introduced into the organism of a hyperimmune animal are not entirely destroyed by the organism, but undergo deep variation in the formation of avisual forms and transition to an apathogenic state. From this arises the question of the nonsterility of immunity in producers of anti-anthrax and precipitating sera.

Investigation of regenerated cultures of anthrax microbes has resulted in establishment of the fact that several strains have pronounced immunogenic properties. Thus the possibility of obtaining from immunosera strains suitable for vaccinoprophylaxis of animals against anthrax is not excluded.

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